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WADC TECHNICAL REPORT 59-175

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A STUDY OF CHEMICAL METHODS FOR QUANTITATIVE MEASUREMENTS OF CATECHOL AMINES

CHARLES H. DuTOIT, M. D.

MASSACHUSETTS GENERAL HOSPITAL
BOSTON, MASSACHUSETTS

APRIL 1959

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CONTRACT No. AF 33(616)-5003

AERO MEDICAL LABORATORY
WRIGHT AIR DEVELOPMENT CENTER
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UNITED STATES AIR FORCE
WRIGHT-PATTERSON AIR FORCE BASE, OHIO

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CONTRACT NO. AF 33(616)-5003
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FOREWORD

This report represents work completed under Contract No. AF 33(616)-5003 on the development of a method for quantitatively measuring catechol amines — adrenaline and noradrenaline -- in urine. The chemical analysis is based on modifications of a well known fluorometric method.

This study was produced under Project 7216 "Biophysics of Acceleration," Task 71712. Captain Stuart C. Bondurant, Acceleration Section, Aero Medical Laboratory, served as contract monitor. The technical assistance of Miss Luba Geller is gratefully acknowledged. The author is indebted also to Miss Mary Ellen Collins for assistance in making fluorescence spectra measurements, to Dr. Robert S. Shaw, Massachusetts General Hospital, for checking the original manuscript of the work reported here, and to Dr. Arnold R. Slonim, Aero Medical Laboratory, WADC, for assistance in editing this material for a Technical Report.

Manuscript released by author September 1958 for publication as a WADC Technical Report.

Author is now deceased. Correspondence pertaining to the material in this report should be addressed to the Director, Chemistry Laboratory, Massachusetts General Hospital, Boston 14, Mass.

ABSTRACT

A method for the estimation of adrenaline and noradrenaline in urine is presented. This method employs an ion exchange resin, Amberlite XE-64, for purification. The simultaneous estimation of the two hormones is accomplished by a multiple filter technique of fluorometry. The selection of appropriate sets of filters is based on a careful study of the fluorescence spectra of the compounds concerned.

The method has been subjected to careful scrutiny as to factors affecting reproducibility and precision of the measurements. Evidence is presented that a high degree of specificity has been attained.

PUBLICATION REVIEW

This report has been reviewed and is approved.

FOR THE COMMANDER:

Andres I. Karstens
ANDRES I. KARSTENS
Colonel, USAF (MC)
Asst. Chief, Aero Medical Laboratory

INTRODUCTION

The objective of this study was the development of quantitative methods for the measurement of adrenaline and noradrenaline* in body fluids. Variations in the production of these two hormones are thought to be of considerable importance in the adaptation of individuals to both physical and emotional stresses. Reliable methods applicable to large scale physiological experimentation are badly needed to test these hypotheses.

A method has been developed combining the modifications of ion exchange purification as first proposed by Bergström and Hansson (1) and the quantitative fluorescence reaction of Lund as modified by Von Euler and Floding (4).

METHODS

Equipment and Reagents

(a) Instruments. The Farrand Fluorometer, Model A, and the Aminco-Bowman Spectrofluorimeter are used in this study.

(b) Optical Filter Systems

The system isolating epinephrine fluorescence predominantly, referred to as the "E" system, is composed of a primary and secondary filter. The primary (or activation) filter consists of a 436 mμ interference filter (Baird Associates or Farrand Optical Co.) or a combination of Corning 5113 and 3389. The secondary filter consists of a Corning 3486; this filter excludes light of wave lengths less than 525 mμ.

The system isolating norepinephrine fluorescence predominantly, referred to as the "N" system, is composed of a primary and a secondary filter. The primary filter consists of a Corning 4308, 9863, and a Wratten 36; this filter combination has a light window between 400 and 420 mμ. The secondary filter consists of a Kodak 75. (The transmission characteristics of this secondary filter are shown in Fig. 1.)

(c) Reagents

Reagent grade chemicals are made up in the following concentrations: hydrochloric acid (0.5N, 1N, and 6N), acetic acid (0.33M and 1.0M), sodium

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* Although the official American terminology of these two catechol amines is epinephrine and norepinephrine, the terms adrenaline and noradrenaline are preferred and used in this report because the compounds involved in the fluorescent reaction are derived from the same base, e.g., adrenochrome and adrenolutine.

hydroxide (1N and 5N), sodium chloride (0.2 gm%), potassium ferricyanide (0.25 gm%) and zinc chloride (0.5 gm%).

Ethylene diamine tetraacetate (EDTA), 0.1M, is prepared by dissolving 37.2 gm of disodium EDTA-$2H_2O$ per liter of water.

Alkali-ascorbic acid is prepared just before use by dissolving 1.0 gm of ascorbic acid in 50 ml of water; 1 ml of this solution is added to 9 ml of 5N NaOH.

Sodium phosphate, 0.5M, buffer. 0.5M Na_2HPO_4 (71 gm/liter) and 0.5M $NaH_2PO_4 \cdot 2H_2O$ (69 gm/liter) are prepared and mixed together to give a pH of 6.2 - 6.3 on a pH meter. A mixture of exactly 2 monosodium to 1 disodium salt solution gives the desired pH.

Sodium acetate, 1M, is buffered to pH 6.2 with 1.0M acetic acid and checked on the pH meter.

Amberlite XE-64 resin (Rohm and Haas Co.) glass wool plus Baker's purified sand are used for the columns.

(d) Standards

The following stock standards are used: epinephrine (adrenaline) bitartrate-U.S.P. primary reference standard (18.2 mg in 1 liter of 0.33M acetic acid; this keeps indefinitely in a refrigerator), and norepinephrine (noradrenaline) bitartrate — Winthrop Stearns Co., N.Y. (20.0 mg in 1 liter of 0.33M acetic acid).

Working standards are prepared by diluting 5 ml of each of the above stock standards to 100 ml with water; this keeps only 3 - 4 days in a cold room (at 4° C). One ml of working standard is equal to 0.5 μg of either amine.

Preparation of Resin (from sodium form)

A 2 to 5 cm layer of dry resin is placed into a 500 ml Erlenmeyer flask, and water is added with shaking to just below the neck of the flask. The contents are allowed to stand one-half hour or more. Excess water is decanted (the supernatant solution will be cloudy at this stage no matter how long the resin stands).

The resin suspension is washed at least twice with 1N HCl, once with water, once with 1N NaOH, once with water again; the washing procedure is then repeated entirely.

It is important to wash the resin in the "H" or acid form until the supernate is nearly clear between washings. After the final washing in the sodium form, a slurry about 5 cm deep is made so that it will draw conveniently into a 10 ml reagent pipet with a broken tip.

Preparation of Columns

Columns with bulb of 250 ml capacity, 35 cm long, and 1 cm in inner diameter are used in this work.

A plug of glass wool is pushed with a glass rod to the constriction of the column. The bulb is well filled with water, and some water is run through to pack the glass wool plug. Some Baker's purified sand is shaken in to make a layer about 1 cm above the glass wool plug. The column is tapped or shaken until the surface of the sand is horizontal.

The resin slurry is added with a 10 ml pipet and the resin is washed down the sides with a wash bottle. The stopcock is opened, drainage is allowed, and the resin is added continually until the resin column is approximately 15 cm long. When the resin has settled completely, a small glass wool plug is added to the top of the column.

(a) Buffered Column Treatment. The water level is drained to the top of the plug. Forty to fifty ml 0.5M phosphate buffer, pH 6.3, is added; drainage is allowed until column shrinkage is complete and the pH of the effluent is 6.3. The column is allowed to stand at this stage overnight.

(b) Acid Phase Column Treatment. The above column is treated with 50 ml 1N HCl instead of buffer and washed with 50 - 100 ml water. At the end of the run, the eluted column may be washed with some 1N HCl and water, and used as the acid phase column for the next test.

(c) Treatment on Day of Test. Excess buffer is drained off and the column is washed with 0.2% NaCl until the effluent pH is around 5.0 to pH paper (takes 50 - 60 ml); the column is now ready for use.

Preparation of Urine Samples

A twenty-four hour urine sample is collected with 10 ml 6N HCl (5 ml con. HCl, 5 ml water); the final pH should be between 1.2 and 3.0. Any sample outside this range is rejected From 50 to 100 ml aliquots are used depending on the 24 hour volume.

For recovery experiments parallel samples are run on parallel columns with 5.0 μg of either epinephrine or norepinephrine added to one sample before being put on the column. The pH is adjusted to 5.0 - 5.1 with 1N NaOH; 100 - 150 ml of water is added, so that the sample is between 0.01 and 0.04 molar with respect to the NaOH needed for neutralization.

The sample is passed through the acid phase column and washed once more with 50 ml 0.2% NaCl. All effluent is saved and washed.

The material is run through the buffered column for 2 - 4 hours and is washed twice: once with approximately 20 ml and once with 50 ml of 0.2% NaCl.

Elution

From 40 to 50 ml of 0.5 N HCl is added and the column is watched closely. The zone of transformation of the resin from the sodium to the hydrogen form can be followed as there is a change in color from the pink of the sodium to the white of the acid phase. The eluate is discarded until about 7.5 cm of the sodium phase remains (2/3 of the column). The eluate is collected from this point on until the column is completely decolorized. With practice this can be accomplished with 25 ml of eluate, although 30 ml is safer for large scale use.

Development of Fluorescent Reaction

A 10 ml glass-stoppered graduated tube is used for sample, blanks and standard, and the following reagents are added in order:

(a) Sample -
1. 0.1 ml 0.5% $ZnSO_4$
2. 3.0 ml eluate equivalent to 5 ml urine
3. 1.0 ml 1M acetate buffer, pH 6.1 - 6.2
4. 1N NaOH is added until pH is approximately 6.0 (err on high side); pH hydrion paper range 5.2 - 6.6 is convenient for this neutralization. Make up to 5 ml mark.
5. 0.1 ml 0.25% ferricyanide; let stand exactly 2 minutes after mixing.
6. 0.5 ml EDTA solution
7. 1.0 ml alkali-ascorbic mixture. Make up to 10 ml mark.
8. Fill cuvette and read on fluorometer.

(b) Unoxidized Blank - It is treated exactly as the sample except that ferricyanide (step 5 above) is omitted.

(c) Reagent Blank - Water is substituted for eluate in step 2 above.

(d) Standard(s) - One ml of working standard plus 2 ml of water are used in step 2 above (1 ml working standard is equal to 0.5 μg of catechol amine).

Measurement

Using filter system E the galvanometer is set at 100 with the adrenaline standard. Standards, samples, and blanks are read in order. The filters are changed to filter system N, and the galvanometer is set at 100 with the noradrenaline standard. The galvanometer readings are taken in the same order.

Calculation

The following simultaneous linear equations are solved for x and y (with corrections made for the readings of the blanks):

$$xA + yC = HF$$
$$xB + yD = HG$$

Where A = Reading Adrenaline Std on E system (=100) minus Reagent Blank.

B = Reading Adrenaline Std on N system minus Reagent Blank.

C = Reading Noradrenaline Std on E system minus Reagent Blank.

D = Reading Noradrenaline Std on N system (= 100) minus Reagent Blank.

F = Reading of Sample on E system minus Eluate Blank on E system.

G = Reading of Sample on N system minus Eluate Blank on N system.

H = Concentration of Standards, usually 0.5 µg/10 ml assay mixture.

x = Concentration of Adrenaline in µg/10 ml.

y = Concentration of Noradrenaline in µg/10 ml.

Since 10 ml assay mixture is equivalent to 5 ml urine, the value of x (or y) X 200 = the concentration of the respective catechol amine in µg/liter of urine.

RESULTS

The isolation or first step of the method consists in adsorption of the catechol amines on a carboxylic acid cation exchange resin, Amberlite XE-64. The columns are washed with dilute saline and the catechol amines subsequently eluted with dilute hydrochloric acid. The type of resin used is particularly suitable for isolation of organic bases. It behaves as a weak acid itself and thus can be buffered to control the pH of the internal environment. When buffered at pH's near neutrality, amphoteric compounds such as neutral amino acids are poorly retained. Elution is easily accomplished with very dilute mineral acids. This method of purification avoids the excess alkalinity which characterizes other methods of isolation of these compounds -- as a result the ecoveries are good. For adrenaline the mean recovery is 93% and for noradrenaline 91%. The 95% confidence limits, based on 30 experiments with each compound, for recoverability are 19.4% for each. Indications are that increased experience on the part of the chemist can narrow this range considerably.

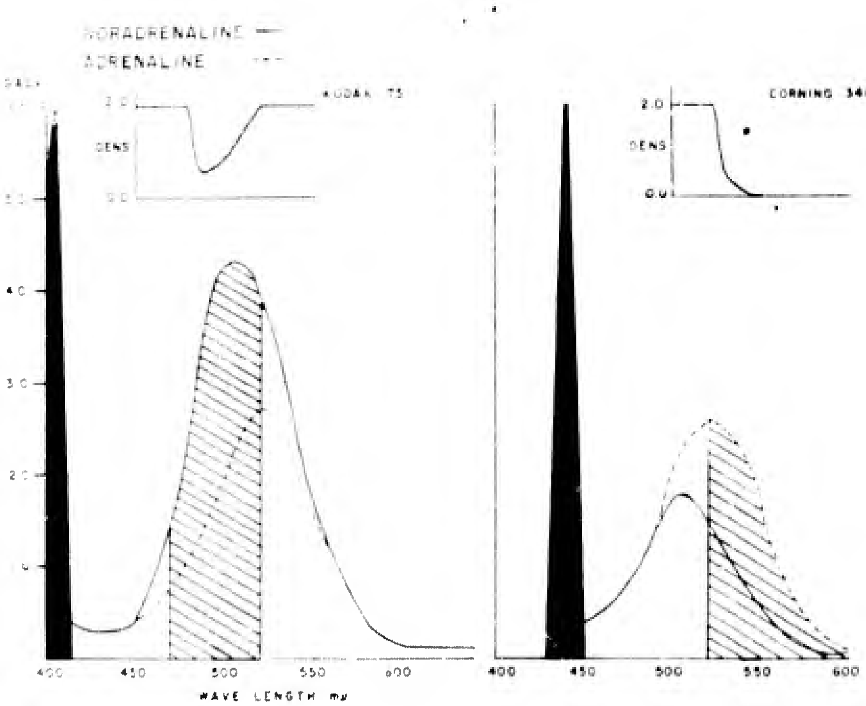

Figure 1 - Emission spectra at conditions simulating the filter system employed with Farrand fluorometer. Optical density of secondary filters as measured by Carey spectrophotometer in inset. Shaded area represents segment of fluorescent spectra of compounds transmitted by secondary filters and is projected portion shown in optical density figure in inset.

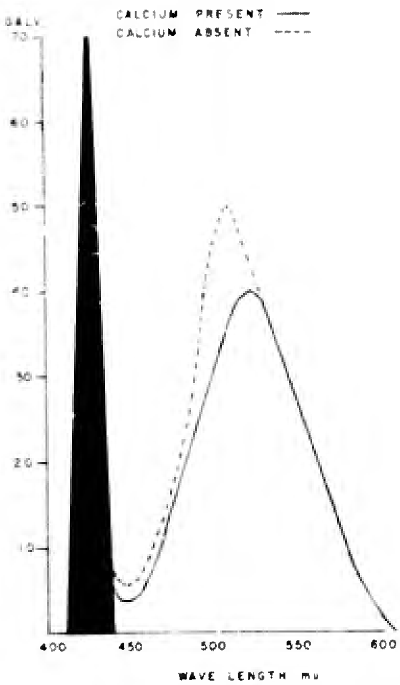

Figure 2 - Effect of calcium chloride, 0.002M, on the emission spectrum of adrenolutine.

The chemical reaction is based on the well-known oxidation of the catechol amines to the adrenochromes and then subsequent rearrangement in alkali to trihydroxyindoles. With the Aminco-Bowman spectrofluorometer, fluorescence spectra of these compounds have been obtained. An important observation has been made, namely, that the presence of heavy metals alters not only the intensity of emitted light but the emission spectra as well (Fig 2). This problem is easily surmounted by the use of ethylene diamine tetraacetate (EDTA) in the second stage of the reaction. By means of the spectral data obtained (Figs 1, 3, and 4) we have been able to make a rational selection of primary and secondary filters for use on filter fluorometers. It is possible to se up optical conditions wherein the fluorescence of adrenaline and noradrenalinederivativesare strikingly different (Fig 5). In this way a binary system is set up and the absolute amounts of each substance can be easily calculated. Of course such a calculation introduces an additional source of error since the error of measurement of one substance affects the other. This objection is somewhat mitigated by the fact that a

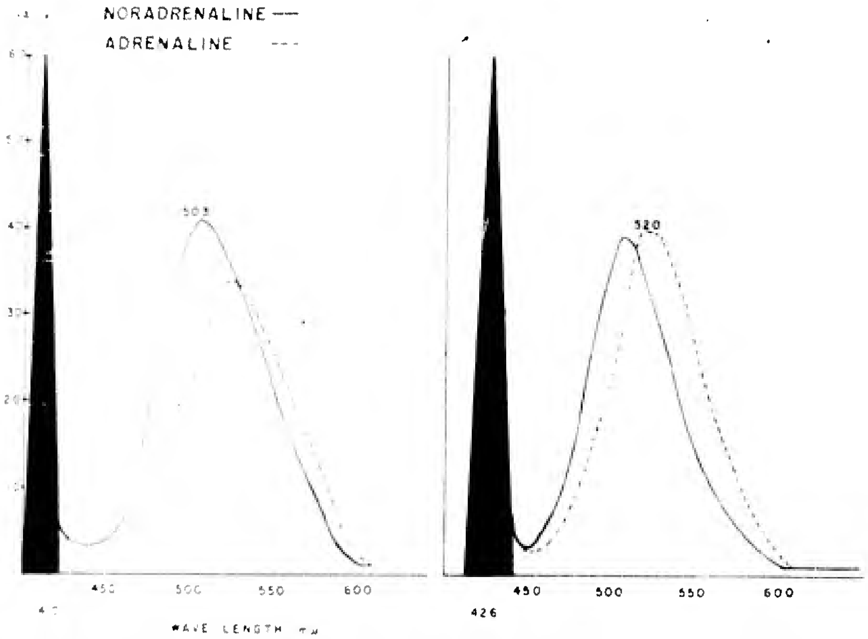

Figure 3 - Emission spectra at respective activation maxima made on Aminco-Bowman spectrofluorimeter with 0.003 resistance. Each compound present in 0.05 μg ml concentration.

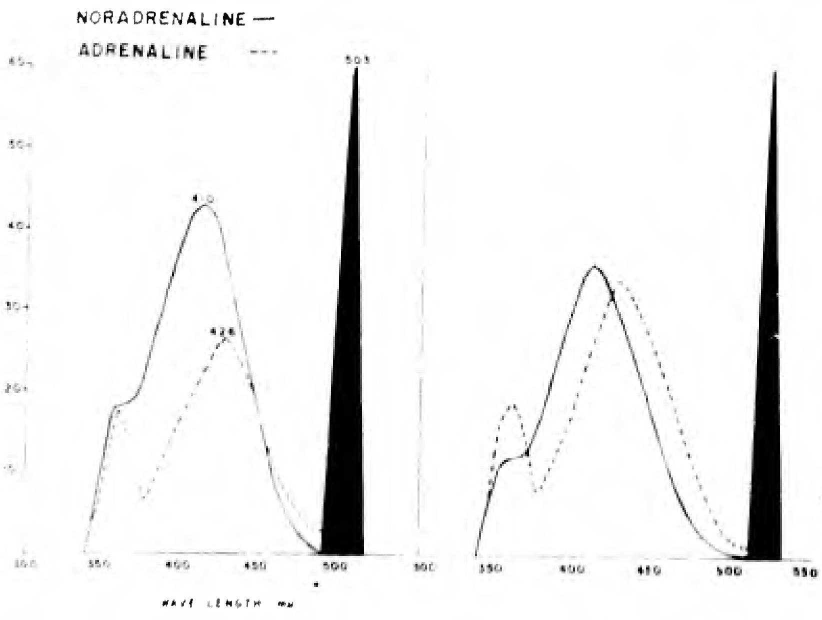

Figure 4 - Activation spectra at respective emission maxima.

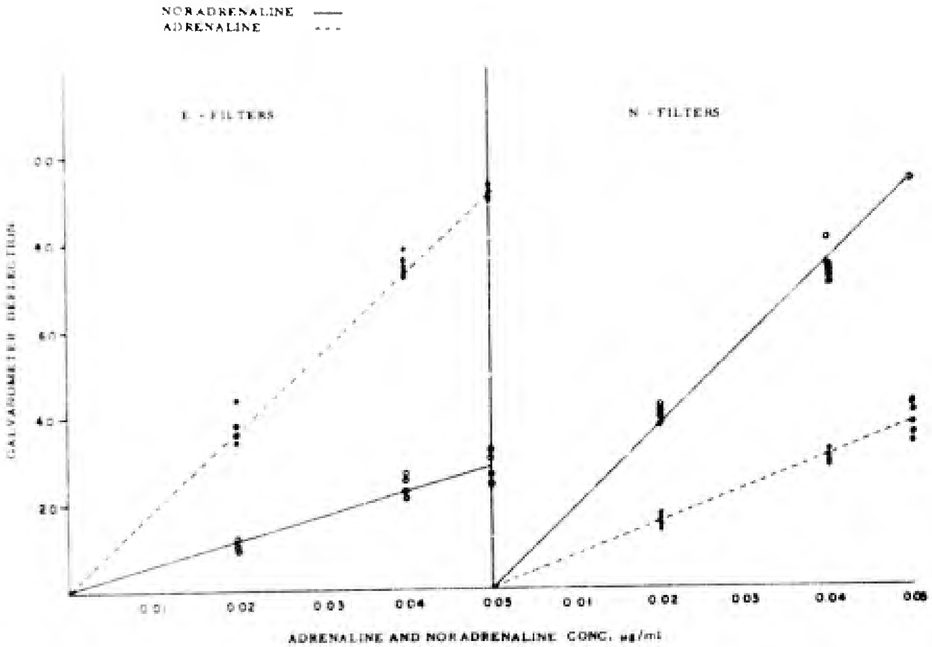

Figure 5 - Standard curves of fluorescence of adrenaline and noradrenaline derivatives with the selected filter systems.

single manipulation is needed in contrast to other methods such as von Euler's differential oxidation. From differential recovery data it appears that the added error introduced by this factor is not serious in the physiological concentrations; it becomes important only where marked excesses of one or the other occurs such as in pheochromocytoma.

DISCUSSION

The specificity of the method has not been rigorously determined. It has not been possible to compare the results against a completely independent analysis for instance bioassay. This has been done by von Euler using a somewhat similar chemical method, and the results are in fair agreement although tending to be somewhat higher by chemical than by biological assay. It can not be assumed that bioassay if lower is more correct.

One of the great objections to fluorometric assay has been the large number of biological compounds that exhibit fluorescence under one condition or another. To date no method of isolation completely eliminates fluorescence obviously not due to oxidized adrenaline or noradrenaline. In fact compounds, possibly indoles, have been isolated on sulfonic acid resins which develop appreciable fluorescence

under the same conditions as the adrenaline family. Small mounts of these appear in the eluate from Amberlite XE-64. However, it has been found that these and a great deal of the "blank" fluorescence can be eliminated by a preliminary passage of the urine through a column of the same resin, Amberlite XE-64, in the hydrogen cycle. This removes impurities that have an affinity for the resin because of other than ionic properties. The buffered columns after elution are washed briefly and used as hydrogen columns in the next test, so that this step does not unduly lengthen or complicate the procedure.

The Aminco-Bowman instrument has also been helpful in establishing appropriate conditions for taking into account the non-specific fluorescence. Eluates in the present method contain a substance or family of substances having some fluorescence with an emission maximum at 465 mμ and an activation maximum of 370mμ. It contributes some fluorescence on the lower wave length filter system employed in the Farrand instrument. This fluorescence is present after treatment with potassium ferricyanide at pH 6, and is present after treatment of non-oxidized eluates with the alkali-ascorbate mixtures (see Fig 6). Therefore, it seems that an appropriate blank is established by omitting the oxidizing step. Older methods used a blank in which the ascorbic acid was omitted; under these conditions the trihydroxindoles rapidly disappear. However the non-specific fluorescent component does likewise. Furthermore, thiochrome which contributes a slight amount of fluorescence with the filter systems employed is formed under these conditions but in neither the total reaction or blank we employ.

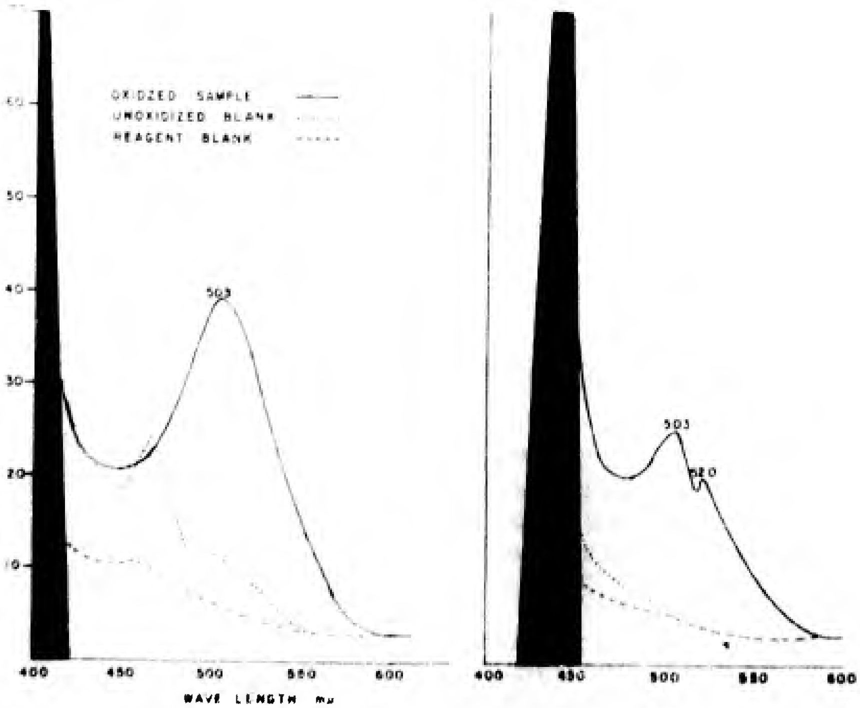

Figure 6 - Emission spectra at activations of 405 and 440 mμ made on eluate from a patient. Calculated values equivalent to 5 ml of urine are 0.081 μg noradrenaline and 0.01 μg adrenaline. Eluate blank and reagent blank are superimposed.

A large number of urine eluates* have been studied with the Aminco-Bowman instruments. In most physiological and pathological states noradenaline exceeds adrenaline by nearly ten fold. Moreover, with respect to general contour and position of maxima, both the emission and activation spectra closely parallel those developed by identical treatment of a pure solution of noradrenaline. In some cases where the ratio of adrenaline to noradrenaline is higher, an inflection point or subsidiary peak at the adrenolutine maximum can be seen (Fig. 6).

Some distortion of the noradrenaline spectra is seen in the lower wave lengths and is consistent with the blank component described above (Fig 6). These observations show that it is unlikely that other components contribute a very large part of the measured fluorescence.

Work is in progress with ion exchange fractionation by the method of Kirshner and Goodall (3). This has already indicated that substances responsible for the blank move much more rapidly through the column than true bases. This observation indicates that a different washing procedure might eliminate most of the remaining blank fluorescence in the routine operation. Preliminary experiments have been promising in this regard.

The most convincing evidence of the validity of the method comes from data on a large number of patients and experimental subjects. The mean values for the first 150 urine samples are adrenaline 4.5 µg and noradrenaline 35.8 µg per 24 hours. These figures are strikingly similar to those reported by von Euler and Floding on bioassay studies of a similar population. With the exception of quinidine and quinine, no drug has been encountered which produces misleading results. These affect the blank also and are not actually responsible for mistakes. Isopropyl noradrenaline, or isuprel, has an identical fluorescence spectrum to adrenaline and is a possible source of error. Of the closely related catechols, dopamine (3-hydroxy tyramine) gives a slight reaction. Dopa (3, 4 dihydroxy phenylalanine) gives fluorescence at lower wave lengths equivalent to 70% of an equivalent amount of noradrenaline, but is not retained on the resin used in this study.

Work has not yet been done on the analysis of catechol amines in blood. However, the principles of the fluorometric technique established here are pertinent to any attempts at chemical assay of these hormones in blood. Very recently work by Cohen and Goldenberg (2) has appeared in which a blood fluorometric method is described based on similar considerations. The normal blood levels described therein are so low that one is forced to question the usefulness of their measurements in physiological experiments on man.

SUMMARY

A multiple filter fluorometric method has been developed which gives a precise measurement of catechol amines in urine. The mean recoveries for adrenaline and noradrenaline are 93% and 91% respectively; the 95% confidence limits for recoverability are 19.4% for each hormone. The technique is simple enough to allow the performance of five or six analyses per technician per day.

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